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Previews

Argonaute Regulation: Two Roads to the Same Destination

Judith Hauptmann[1] and Gunter Meister[1,*]
[1]Biochemistry Center Regensburg (BZR), Laboratory for RNA Biology, University of Regensburg, Universitätsstrasse 31, 93053 Regensburg, Germany
*Correspondence: gunter.meister@vkl.uni-regensburg.de
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Reporting recently in *Nature* and *Nature Structural and Molecular Biology*, Shen et al. (2013) and Smibert et al. (2013) uncover mechanisms for Argonaute (Ago) protein regulation. Smibert et al. find that microRNA availability controls Ago levels, and Shen et al. show that epidermal growth factor receptor-mediated Ago2 phosphorylation affects Ago loading.

Small RNAs such as microRNAs (miRNAs) or short interfering RNAs (siRNAs) associate with Ago proteins and regulate cellular processes in many different species. miRNAs are generated from hairpin-structured precursors by the consecutive action of the two RNase III enzymes Drosha and Dicer. Following these cleavage steps, a single-stranded mature miRNA is loaded onto an Ago protein and the RNA-induced silencing complex (RISC) is formed. In animals, miRNAs guide RISC to partially complementary sequences typically located in the 3′ untranslated region (UTR) of target messenger RNAs (mRNAs). As a consequence, the mRNA is translationally silenced and/or exonucleolytically degraded (Huntzinger and Izaurralde, 2011). siRNAs are generated from long double-stranded RNAs, which can originate either from exogenous (synthetic siRNAs, viral RNAs, etc.) or endogenous (long hairpins, sense-antisense transcription) sources. Similar to miRNAs, Dicer processes these long RNA precursors into siRNAs, which are then loaded onto an Ago protein. Ago-associated siRNAs often recognize fully complementary target RNAs, and a catalytically active Ago protein (e.g., Ago2 in mammals) then cleaves the target RNA sequence-specifically (Kim et al., 2009). Ago proteins are at the heart of both small RNA pathways, and therefore Ago protein levels, as well as their functions, are highly regulated. Two recent publications, Smibert et al. (2013) in *Nature Structural and Molecular Biology* and Shen et al. (2013) in *Nature*, now provide insights into how Ago protein levels and Ago loading processes are controlled (Figure 1).

To explore the regulation of Ago, Smibert et al. (2013) used the *Drosophila* system in which the miRNA and the siRNA pathways are clearly distinct. Compared to mammals, in which there is only one Dicer and in which both miRNAs and siRNAs can be loaded onto all Ago proteins, *Drosophila* Dicer1 (Dcr1) produces miRNAs that are bound by Ago1, and Dicer2 (Dcr2) generates siRNA, which associates with Ago2. Using genetic in vivo approaches combined with immunofluorescence staining of Ago1, Smibert et al. (2013) show that genetic inactivation of miRNA processing factors such as Dcr1 leads to reduced Ago1 protein levels (Figure 1). This is not due to transcriptional regulation but rather to protein degradation because inhibition of the ubiquitin-proteasome system stabilized Ago1 under these conditions. Interestingly, Ago2, the siRNA effector protein, is not controlled by the levels of siRNAs in *Drosophila*, suggesting that the two small RNA pathways employ different mechanisms to control Ago protein levels. Smibert et al. (2013) also analyzed human Ago protein expression. They find that Ago2 levels are reduced in Dicer-deficient (and therefore also miRNA-lacking) mouse embryonic fibroblasts. They show that this effect is also caused by proteasome-mediated protein degradation and can be rescued by the transfection of small RNAs. In RNA interference experiments, it is generally observed that siRNAs are efficiently loaded onto Ago2, suggesting the existence of a pool of free Ago2 that can efficiently take up the transfected small RNAs. However, the work by Smibert et al. (2013) now demonstrates that such stable small RNA-free human Ago2 pools might not exist and that extra siRNAs might either compete with endogenous miRNAs for loading or stimulate Ago2 synthesis by unknown mechanisms.

Whereas Smibert et al. (2013) focused on homeostatic regulation of Ago, the work of Shen et al. (2013) examined the regulation of Ago loading under hypoxic conditions and uncovers a mechanism for its regulation by phosphorylation (Figure 1). Using biochemical approaches, Shen et al. (2013) find that Ago2 interacts with the epidermal growth factor receptor (EGFR) in cultured human cells. Under hypoxic conditions, often found in the center of solid tumors, EGFR is a well-characterized oncogene. Because miRNAs are also often involved in cancer, the interaction of EGFR with Ago2 prompted the authors to analyze miRNA expression upon EGFR knockdown under normoxic or hypoxic conditions. Indeed, they find that the expression of a specific number of miRNAs depends on EGFR under hypoxic conditions. Based on the current literature, most of these miRNAs seem to have tumor-suppressive functions. Furthermore, the EGFR effect on miRNA expression requires a functional kinase domain. Consequently, Shen et al. (2013) find that Ago2 is phosphorylated at tyrosine 393 (Y393) and that this phosphorylation is mediated by EGFR. Interestingly, phosphorylation of Ago2 at Y393 impairs Dicer interaction, and thus the loading of a specific subclass of miRNAs onto Ago2 under hypoxic conditions (Figure 1). Why is only a limited number of miRNAs affected by interfering with the Ago2-Dicer interaction? Shen et al. (2013) analyzed the precursor

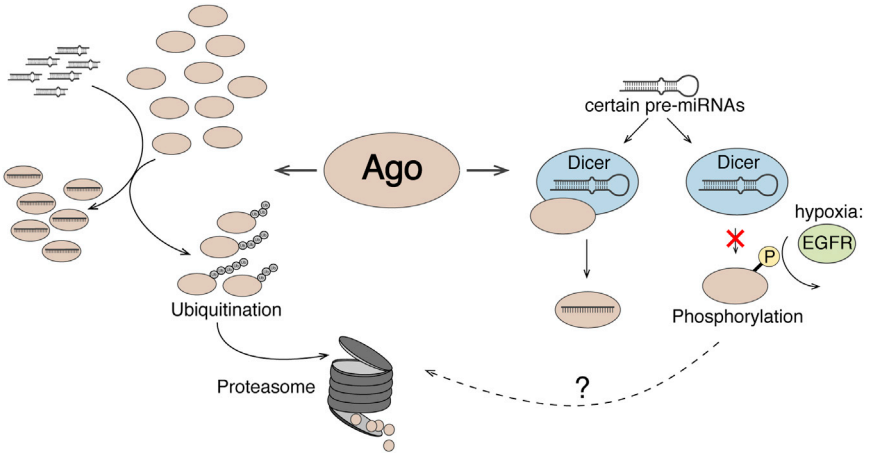

Figure 1. Regulation of Ago Proteins by miRNA Availability or Tyrosine Phosphorylation
Ago protein levels are regulated by miRNA availability (left), whereas Ago loading with a specific subset of miRNAs is controlled by tyrosine phosphorylation (right). In *Drosophila* and mammals, reduction in miRNA levels results in Ago protein reduction by ubiquitination-mediated degradation by the proteasome (left). Under hypoxia conditions, EGFR phosphorylates Ago2 on Y393. A subset of specific, large loop-containing miRNA precursors is not processed under these conditions. During the RISC loading process, a phosphorylated Y393 on Ago2 might interfere with the transfer of the processed miRNA to Ago2, and thus these miRNAs are not loaded (right).

structures of the affected miRNAs and found that these miRNAs contain larger loop structures. The authors therefore suggest that phosphorylation of Y393 specifically influences the maturation of these large loop-containing miRNA precursors in a Dicer-dependent manner. Using a polyclonal antibody directed specifically against Y393 phosphorylated Ago2, the authors analyzed Ago phosphorylation status in breast cancer patient samples. With this approach, they were able to correlate Y393 Ago2 phosphorylation with poor patient survival, leading to a model in which hypoxia-induced upregulation of EGFR results in Ago2 phosphorylation and downregulation of several tumor suppressor miRNAs due to impaired Ago2 loading.

Ago proteins are key factors in the miRNA pathway, and therefore it is not surprising that these proteins are highly regulated. When cells react to specific stimuli or changing conditions, signaling cascades help to establish new gene expression programs. miRNAs are important pieces in such gene expression networks, and their regulation can be achieved through the phosphorylation of Drosha (Tang et al., 2010), TRBP (Paroo et al., 2009), or Ago proteins (Horman et al., 2013; Rüdel et al., 2011; Zeng et al., 2008) proteins. Several additional phosphorylation sites have been mapped in different human Ago proteins (Rüdel et al., 2011). It is tempting to speculate that a number of signaling pathways, in addition to EGFR, also influence Ago function. It is also not clear yet whether different Ago proteins are differentially phosphorylated and which kinases are required for these modifications. In addition, it has been shown that other posttranslational modifications such as hydroxylation can affect Ago protein stability (Qi et al., 2008), suggesting that Ago protein levels are regulated not only by miRNA availability but also by a range of posttranslational modifications. Future work will unravel the impact of posttranslational modifications on the miRNA pathway, as well as its consequences for diseases such as cancer.

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